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A Quantitative Assay for DNA–RNA Hybrids with DNA immobilized on a Membrane

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An improved method for the formation of DNA–RNA hybrids is described. The procedure involves immobilizing denatured DNA on nitrocellulose membrane filters, hybridizing complementary RNA to the membrane-fixed DNA, and eliminating RNA "noise". Denatured DNA and hybridized RNA remain on the filter throughout the procedure. Unpaired RNA is removed by washing, and RNA complexed over short regions is eliminated by RNase treatment.

Many samples can be easily handled, permitting kinetic and saturation studies. Large amounts of DNA can be loaded on a filter without interfering with the efficiency of the annealing reaction. Moreover, the "noise" can be depressed to a level (0·003% of the input RNA) permitting the identification of small regions of DNA complementary to a given RNA species. The results are quantitatively more certain than annealing in liquid, since the competing DNA renaturation reaction is suppressed.

1. Introduction

The usefulness of hybridizations between DNA and radioactive RNA as a test for complementarity has stimulated the search for procedures which combine reliability and convenience. Initially, Hall & Spiegelman (1961) used annealing in solution and equilibrium density-gradient centrifugation (Meselson, Stahl & Vinograd, 1957) in preparative rotors for hybrid detection.

To exploit this technique for the identification of small (less than 0·1%) complementary segments, ribonuclease treatment was introduced (Yankofsky & Spiegelman, 1962*a*) to eliminate low levels of adventitious contamination. Here advantage is taken of the resistance to ribonuclease of RNA complexed to DNA. This permitted the detection and measurement of the proportion of DNA complementary to the two ribosomal RNA components (Yankofsky & Spiegelman, 1962*b*,1963) and the S-RNA molecules (Giacomoni & Spiegelman, 1962; Goodman & Rich, 1962).

All of the above studies used density-gradient separation of hybrids; this required lengthy centrifugations. The full realization of the potentialities of the hybridization technique clearly required the design of a procedure less costly in time and expensive equipment. The first success came with the introduction by Bautz & Hall (1962) of nitrocellulose columns to which glucosylated DNA was attached. This method was quickly generalized by Bolton & McCarthy (1962), who recognized that mechanical immobilization *per se* would suffice; and from this concept they developed agar columns containing denatured DNA trapped in the solidified gel. Britten (1963) fixed DNA by ultraviolet irradiation to synthetic polymers which were then employed as columns for RNA hybridization. Most recently, the ultimate in convenience and capacity for handling many samples was provided by the discovery (Nygaard &

Hall, 1964) that nitrocellulose filters strongly adsorbed single-stranded DNA along with any hybridized RNA.

To appreciate the purpose of the present investigation, it is important to recognize that hybridization involves both hybrid formation and hybrid detection. Except for the two column procedures, all the methods described anneal the DNA and RNA in solution and then assay the amount of hybrid complex. Hybridization in solution has one obvious disadvantage, stemming from the fact that RNA–DNA formation must compete with the re-formation of the DNA–DNA complexes. The existence of these competitive interactions can introduce serious errors, particularly in experiments determining saturation plateaux.

In certain instances the complications of annealing in solution can be surmounted by incubating at temperatures well below the melting temperature (T_m) of the DNA, a strategy used by Yankofsky & Spiegelman (1963, and unpublished observations). However, low temperature is not a universally available solution. RNA molecules possessing an extensive secondary structure will not hybridize until their own melting temperature is approached, a situation encountered with S-RNA (Giacomoni & Spiegelman, 1962).

In principle, immobilization of the DNA during the hybridization provides a logical method of avoiding these unwanted interactions. The obvious answer was to fix the denatured DNA irreversibly to nitrocellulose membrane filters, and to carry out the hybridization on the filters. This should serve to eliminate, or greatly reduce, DNA–DNA interactions while retaining the almost indispensable convenience of the filter method. To these advantages can be added RNase treatment to eliminate low levels of contamination with unpaired RNA, which can become crucial in many types of investigations.

After exploring several more involved procedures, it was discovered that irreversible fixation of DNA to nitrocellulose membranes was readily achieved by thorough drying at moderate temperatures. The result is a simple and conveniently flexible method of hybrid assay possessing a vanishingly small noise level combined with high accuracy. It is the primary purpose of the present paper to provide the details and describe the use of the resulting procedure.

2. Materials and Methods

(a) *Bacterial strains*

Thymine and uracil auxotrophs of *Bacillus megaterium*, strain KM, were isolated and kindly supplied by Dr J. T. Wachsman. An auxotrophic derivative of *Escherichia coli* $15T^-$ requiring histidine and uracil as well as thymine was used to prepare labeled *E. coli* RNA and DNA.

(b) *Media and buffers*

A basal medium (Mangalo & Wachsman, 1962) supplemented with 20 μg/ml. of uridine or 5 to 10 μg/ml. of thymidine was used. The phosphate concentration was dropped from $2{\cdot}4\times10^{-2}$ M to 1×10^{-4} M for ^{32}P incorporation.

SSC buffer contains 0·15 M-NaCl and 0·015 M-sodium citrate (pH 7·0). 1/100 SSC, $2\times$SSC and $6\times$SSC are buffers containing one one-hundredth, two times and six times these concentrations, respectively.

(c) *Enzymes*

The enzymes used in this study were purchased from the following companies: 5 times crystallized RNase, Sigma Chemical; electrophoretically pure DNase, Worthington Biochemical; lysozyme and bovine serum albumin, Armour Pharmaceutical; pronase, Calbiochem. The RNase was heated to 90°C for 10 min to remove DNase activity.

(d) *Incorporation of ^{32}P and isolation of RNA*

The uracil-requiring strain was grown overnight to late log-phase in basal medium supplemented with uridine. The cells were washed once in basal medium lacking phosphate and resuspended in a medium containing 1×10^{-4} M-PO_4 at an O.D.$_{660}$ of about 0·01. When the cells had reached an O.D.$_{660}$ of 0·05, 50 μC/ml. pyrophosphate-free, neutralized [^{32}P]orthophosphate was added to the culture and the cells were allowed to grow for 2 doublings. The culture was then made 0·024 M in [^{31}P]orthophosphate and growth was allowed to occur for one more generation. The cells were harvested, concentrated, converted to protoplasts and the RNA extracted as described by Yankofsky & Spiegelman (1962*a*,*b*). The RNA prepared by this procedure had an initial specific activity of about 2×10^6 cts/min/μg.

(e) *RNA purification*

The RNA was freed from DNA and other contaminating phosphorus compounds in the following way. To the RNA solution in 0·05 M-PO_4 (pH 6·8) was added 20 μg/ml. of electrophoretically purified DNase (Worthington Biochemicals) and the mixture was incubated at room temperature for 10 min. The RNA was then loaded on a column of methylated albumin coated on kieselguhr (MAK†) that had been previously washed with 25 to 50 vol. of 0·05 M-phosphate buffer. After loading, the column was again washed with a similar volume of 0·05 M-phosphate buffer, and then the RNA was eluted from the column with NaCl gradients ranging from 0·1 to 1·5 M in the same buffer. The DNase and chromatography procedures were repeated until the counts alkali-stable material were less than 0·05%.

(f) *^3H labeling and DNA isolation*

Thymidine-requiring cells (*B. megaterium*) were grown to late log-phase in basal medium supplemented with 20 μg/ml. of thymidine. A portion of this culture was inoculated into basal medium containing tritiated thymidine (1 μC/7 μg/ml.) at an initial O.D.$_{660}$ of 0·01. The cells were allowed to grow until the O.D.$_{660}$ reached 0·6, whereupon the cells were harvested, concentrated and converted to protoplasts; the DNA was extracted by Marmur's procedure (1961). An additional step included was treatment with pronase to remove RNase. The pronase used was self-digested for 2 hr at 37°C, after which it was added at a level of 50 μg/ml. to the DNA and incubated for 2 hr at 37°C. The solution was then made 0·5% in Dupanol and deproteinized with chloroform until no detectable RNase activity remained. The latter was monitored by 20-hr assays at 37°C with radioactive RNA as the substrate. Ethanol precipitations were used throughout. The final DNA precipitate was resuspended in 1/100 SSC and extensively dialyzed against this buffer.

(g) *Denaturation of DNA*

Native DNA preparations were denatured by alkali by bringing the pH of the solution to 13, and after standing for some 10 min, the solution was neutralized. Denaturation was monitored by following the increase in O.D.$_{260}$. Sedimentation velocity analysis also confirmed the single-strandedness of the DNA. Denatured DNA was kept in 1/100 SSC at 100 μg/ml.

(h) *Hybridization with immobilized DNA*

The procedure, in outline, consists of: binding the DNA to nitrocellulose membrane filters (type B-6, coarse, of Schleicher & Schuell); hybridizing RNA to the fixed DNA; and removing RNA "noise", i.e., unpaired RNA and RNA complexed over short regions. The details of each step are presented below.

Step I. Immobilization of denatured DNA on nitrocellulose membrane filters

Denatured DNA solutions were diluted to 5 ml. with $2\times$SSC and passed through a membrane filter (presoaked in $2\times$SSC for 1 min and washed with 10 ml. of the same

† Abbreviations used: MAK, methylated albumin kieselguhr; TCA, trichloroacetic acid.

buffer), then washed with 100 ml. of 2×SSC. The DNA filters were subsequently dried at room temperature for at least 4 hr and at 80°C for an additional 2 hr in a vacuum oven. In some cases, the procedure was carried out with 6×SSC. Prior drying at low temperature was instituted to avoid renaturation of the DNA in the early stages. Filtration of the DNA should be carried out at moderate speeds, particularly with smaller DNA fragments.

Step II. Hybridization

Hybrids were formed by immersing the DNA filters in scintillation vials containing 5 ml. of [^{32}P]RNA in either 2×SSC or 6×SSC as specified. Annealing was generally carried out at 66°C without shaking, after which the vials were chilled in an ice bath. The volume can be cut down to 0·5 ml., if found convenient, by using small tubes and rolling up the filters.

Step III. Elimination of RNA "noise"

The filters were removed from the hybridization fluid and *each side* was washed with 50 ml. of 2×SSC by suction filtration. RNA not completely complexed is destroyed by immersing the filters for 1 hr at room temperature in 5 ml. of 2×SSC containing 20 μg/ml. of heated pancreatic RNase. After RNase treatment, the vials were again chilled and the filters were rewashed on each side as described above. Finally, the filters were dried and counted in a Packard Tri-Carb scintillation counter.

It should be remarked that the RNase treatment must be carried out in 2×SSC, whereas the other steps may be carried out at the higher (6×SSC) buffer concentration.

(i) *Hybridization with DNA in solution*

Hybrids were formed in liquid medium and, after the incubation, the tubes were chilled and the contents collected on membrane filters, followed by washing with 100 ml. buffer (2×SSC or 6×SSC). The filters were then placed, without drying, in 5 ml. of a 2×SSC solution containing 20 μg/ml. of boiled pancreatic RNase. This is then followed by washing with 50 ml. of 2×SSC on each side. It should be noted that no DNA is lost from a filter during the enzyme treatment in 2×SSC or the subsequent washing.

3. Results

Knowledge of the proportions of DNA and RNA in the complex is essential for quantitative interpretation. In all the experiments to be described, ^{3}H-labeled DNA and ^{32}P-labeled RNA are employed. This permits us to monitor with certainty the amounts of the two reactants on the membranes at every stage of the procedures used.

(a) *Retention of DNA by membrane filters*

It was first necessary to check how well the fixed DNA withstands the various steps required for the hybridization and "noise" elimination with RNase. Table 1 demonstrates that single-stranded DNA is retained by the membrane filters under conditions of hybridization and no detectable DNA is lost during any of the subsequent steps. The fact is, as measured by the ^{3}H (counts per minute) retained, no detectable DNA is lost during the RNase treatment in 2×SSC even without prior drying of the filter. Finally, it can be seen (sample 2, Table 2) that shaking during hybridization does not lead to significant removal of DNA from the membrane filters.

Because of its possible convenience, an examination was made of loading the DNA on the filters, not by filtration, but by spotting a known volume of a DNA solution in 2×SSC on a dry filter, allowing it to spread, and then to dry. Here, thorough drying of the filters is essential if the DNA is to be retained at all. Even with this precaution, the spotting method fails to give a completely irreversible fixation. Some DNA is lost with time (about 0·8%/hr at 66°C) during hybridization at elevated temperatures.

TABLE 1

Retention of DNA by nitrocellulose membrane filters

Sample no.	Treatment of the DNA filters	Percentage DNA remaining on the filter	
		6×SSC	2×SSC
1	Incubate at 66°C without shaking	97, 98	101, 97
2	Incubate at 66°C with shaking	100	101
3	Incubate at 66°C without shaking; wash, RNase, wash	99	99

12 μg of [^{3}H]DNA (500 cts/min/μg) were loaded on membrane filters and washed with 100 ml. of the indicated buffer. The DNA filters were dried at room temperature overnight, then at 80°C for 2 hr. They were then incubated in the same buffer for 24 hr at 66°C. The filters were then either rinsed in fresh buffer or carried through the washing and RNase treatments as described in the Materials and Methods section. Each value is an average of three trials with a 2σ of 5%.

TABLE 2

Elimination of RNA noise

Sample no.	Salt		Radioactive material remaining (cts/min)	Percentage input radioactive material remaining (cts/min)
1	2×SSC	– – – – – –	13,838	1·8
2	2×SSC	wash	290	0·059
3	2×SSC	wash; RNase; wash	25	0·0033
4	6×SSC	wash; RNase; wash	108	0·0076
5	6×SSC	wash; RNase; wash (50 μg DNA; 0°C; 6 hr)	99	0·0066
6	TMS†	– – – – – –	81,038	10·0
7	TMS†	wash	23,528	2·9

Unloaded or DNA-containing filters were immersed in buffer containing 2 μg RNA (400,000 cts/min/μg) for 24 hr at 53°C, except for samples 4 and 5. These latter samples were incubated in buffer containing 10 μg RNA (100,000 cts/min/μg) for 6 hr at 66°C. All filters were removed from the RNA solutions and some were washed and/or treated with RNase as outlined in Materials and Methods.

† TMS = 0·3 M-NaCl; 0·005 M-$MgCl_2$; 0·001 M-tris (pH 7·3).

Further, the rate at which DNA is lost is greatly increased (about 3%/hr at 66°C) when the system is shaken during hybridization. Finally, the amount of DNA which can be loaded on a filter is much more limited than with the filtration procedure.

(b) *Elimination of unpaired RNA*

An obvious advantage conferred by fixing the DNA on the filter is the ease with which contaminating RNA is removed. As may be seen from Table 2, simply lifting the membrane out of the hybridizing mixture leaves 98% of the non-complexed RNA behind. The washing procedure brings the contamination down to 0·06% of the input and the RNase treatment reduces this further to 0·003%. Fixed DNA on

the filter has no effect on the capacity to remove non-hybridized RNA. It should be noted from samples 6 and 7 of Table 2 that the presence of magnesium is to be avoided if low contamination levels are desired.

Table 3 compares the amount of radioactive material (in counts) which survives the purification procedure at different levels of RNA and DNA inputs held for various times and temperatures. The actual counts finally observed for the filters are

TABLE 3

Some parameters affecting RNA noise level

Sample no.	μg DNA	μg RNA	Temp. (°C)	Time (hr)	RNA (cts/min) on filter
1	50	2	4	6	37
2	50	10	4	6	62
3	50	50	4	6	94
4	5	10	4	8	53
5	0	10	66	5	36
6	0	10	66	10	71

The standard procedure using 6×SSC was employed to prepare DNA filters. The filters were immersed in 6×SSC for the indicated times, then removed, washed, treated with RNase and rewashed as usual. DNA = 5,000 cts/min/μg; RNA = 100,000 cts/min/μg.

recorded in the last column. They are all less than 100 counts per minute with input counts ranging from 2×10^5 to 5×10^6 cts/min. Increasing the input of RNA by 25-fold resulted only in a 2·5-fold increase in residual counts. The presence of DNA at non-complexing temperature does not increase the contamination. The procedure is clearly effective in eliminating non-complexed RNA.

(c) *Modification of the Nygaard–Hall technique to lower noise level*

We wanted to compare hybridizations carried out with DNA in solution with others in which the DNA is immobilized. The Nygaard–Hall (1963) method omits RNase and, depending on the amount of RNA, has a noise level ranging between about 0·1 and 1% of the input. This amount of contamination with unpaired RNA was not important, since their experiments involved comparatively massive amounts of hybrid formation. It was, however, too high for our purpose, and we consequently introduced (Materials and Methods section) the RNase step *after* collecting the hybrids formed in liquid on the membrane. Table 4 shows that this modification lowers the noise level from 0·08 to 0·003%.

Another useful fact is recorded in Table 4. Note that RNase treatment *before* collection of the complex on the filters *increases* the "noise" from 0·08 to 0·2%. The RNase, being a basic protein, tends to remain on the membrane when the treated complexes are filtered, and it adsorbs small fragments of RNA which would otherwise be washed away.

Table 5 dramatically illustrates the difficulty introduced by the presence of any basic protein at the filtration step. Note that with either lysozyme or methylated albumin present, between 37 and 75% of all the input RNA appears as "noise" on the filter. The non-basic proteins do not have this unfortunate effect.

TABLE 4

RNA noise level of liquid hybridization techniques

Sample no.	Method for RNA noise elimination	Percentage of input of RNA remaining
1	No RNase	0·0718
2	RNase before filtration	0·2005
3	RNase after filtration	0·0034

Reaction mixtures containing 50 μg DNA and 50 μg RNA (600,000 cts/min/μg) were made up at room temperature in 2×SSC and collected by filtration on nitrocellulose membrane filters. RNase (20 μg/ml.) treatment was carried out at room temperature for 1 hr. All filters were washed with 100 ml. of 2×SSC after collection of the reaction mixtures. Sample 3 was additionally washed with 100 ml. of 2×SSC after RNase treatment (Materials and Methods, section (h)).

TABLE 5

Effect of proteins on the RNA noise level

Sample no.	Protein addition	Percentage of input RNA remaining
1	None	0·108
2	None	0·071
3	Lysozyme	74·9
4	Methylated albumin	37·9
5	Bovine serum albumin	0·061
6	Pronase	0·029
7	DNase	0·043

Proteins (20 μg/ml.) were added to 5 ml. of 2×SSC solutions containing 50 μg each of RNA and DNA and the mixtures were incubated at room temperature for 1 hr. The reaction mixtures were passed through membrane filters and washed as described for samples 1 and 2 of Table 4 (RNA = 120,000 cts/min/μg).

(d) *Retention of DNA by membrane filters as a function of DNA size*

In most of our experiments, the DNA used for hybridization had an $S_{20,w}$ value of about 35 s. There are situations where it is desirable, or necessary, to use smaller DNA fragments. It was, therefore, of interest to see whether size influenced the capacity of the membranes to retain DNA. Accordingly, sheared and unsheared DNA were combined and fragments of different size classes were separated on a sucrose gradient. Each fraction collected was then filtered onto membrane filters and the amount of DNA retained on the filters compared with the total amount of DNA in each sample.

The reversibility of the fixation was tested by carrying each loaded membrane through all the stages of the hybridization tests. The results of these experiments are summarized in Fig. 1. The lowest curve (cts/min) gives the size distribution of the ^{3}H-labeled DNA used. A comparison was made of retention before (not incubated) and after (incubated) the hybridization steps employing two buffer concentrations. Three facts are evident: (1) more DNA is retained by the filters when 6×SSC is used

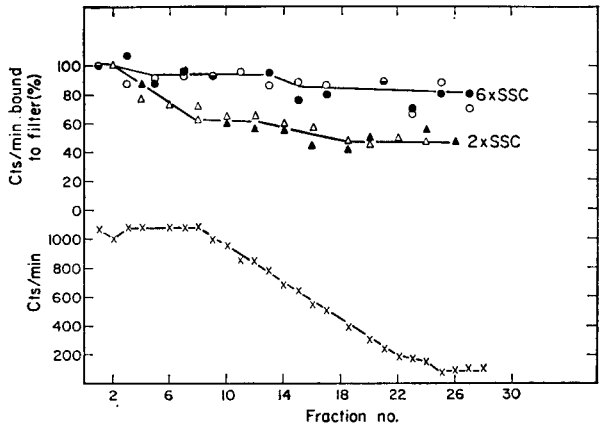

FIG. 1. Retention of DNA as a function of size. 0·5 ml. native DNA (725 μg/ml. in 1/100 SSC) was sheared by passing it 10 times through a Tomac 1 ml. disposable syringe. The sheared DNA was mixed with 0·5 ml. unsheared DNA and heated to 95°C for 5 min. The denatured DNA was layered over a 2·5 to 15% sucrose gradient in 0·1 M-NaCl–5×10^{-3} M-$MgCl_2$–0·01 M-tris (pH 7·3) and spun at 25,000 rev./min for 8 hr at 19°C in the SW25 head of the Spinco model L centrifuge. Samples collected and assayed for TCA-precipitable radioactive material are indicated by the lower curve (—×—×—). (—●—●—, —▲—▲—) Percentage retention on filtration; (—○—○—, —△—△—) percentage of the DNA remaining after the hybridization and purification steps. The results included in Fig. 1 are corrected for losses of radioactive material (in counts) during TCA precipitation; about 20% more radioactive material is retained on membrane filters when 6×SSC is used than when TCA is used. Control experiments revealed that this was not a case of the TCA absorbing radioactive disintegration substances, but a case of differential DNA retention.

than when 2×SCC is used; (2) larger pieces are retained somewhat more readily than small ones; and (3) once fixed, no DNA, big or small, is lost during the hybridization and washing steps.

(e) *Availability of the DNA for hybridization*

The next question we wanted to resolve was whether the sequences of the immobilized DNA were as freely available for hybridization as in solution. Since the saturation plateau for ribosomal RNA had already been carefully examined, it was

TABLE 6

Availability of DNA for hybridization

Hybridization technique	Average complexed (cts/min)	Percentage DNA hybridized ($\bar{x} \pm 2\sigma$)
DNA in solution	11,711	0·314 ± 0·027
Membrane-fixed DNA	1702	0·315 ± 0·008
CsCl		0·303

The annealing in liquid was carried out in 6×SSC, using 40 μg[^{3}H] DNA (5000 cts/min/μg) and 40 μg[^{32}P]RNA (100,000 cts/min/μg) in 1·5 ml. Hybridization was performed at 43°C for 12 hr; subsequent steps follow the procedure outlined in Materials and Methods.

The membrane-fixed DNA technique employed a filter containing 5 μg DNA (5000 cts/min/μg) in a 6×SSC solution containing 10 μg RNA (100,000 cts/min/μg). Hybridization was carried out at 66°C for 8 hr. Purification of the hybrid is described in Materials and Methods. Each number is the average of three experiments ± 2σ.

adopted as a useful test system. Comparisons were made between annealing with DNA in solution and with membrane-fixed DNA. To minimize interference with DNA–DNA interactions, the liquid hybridizations were carried out at 43°C as had been done previously (Yankofsky & Spiegelman, 1963). In all cases, the ratio of RNA to DNA employed was sufficient to attain the saturation plateau, and the times required were determined from preliminary kinetic experiments. Table 6 summarizes the results obtained with dissolved and immobilized DNA, each number representing the average of three determinations. The saturation value obtained previously (Yankofsky & Spiegelman, 1963) by the CsCl density-gradient method with the same biological materials is also recorded. The average plateau values of all four are in agreement, indicating that immobilization of the DNA does not interfere with its capacity to hybridize.

(f) *Capacity of membranes to retain DNA and availability of the DNA for hybridization at low and high DNA inputs*

The capacity of the membranes to retain DNA at different levels of input was examined along with the capacity of the fixed DNA to pair with ribosomal RNA. The results obtained with DNA inputs up to 250 μg per filter are summarized in Fig. 2. It is clear that, within the range tested, the capacity of the membranes for

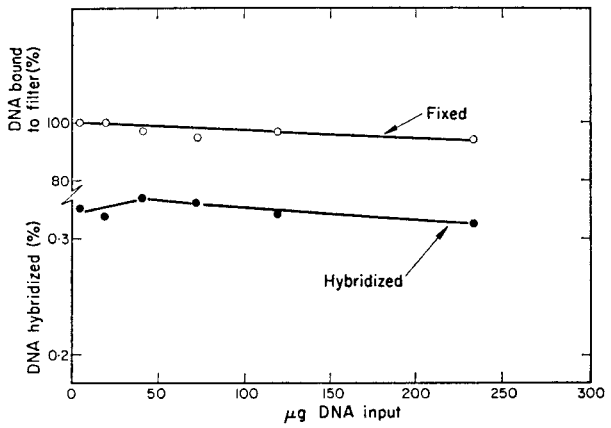

FIG. 2. Filter capacity and availability for hybridization at different levels of DNA. DNA filters were prepared in 2×SSC. Hybridizations were carried out in 0·8 M-NaCl and 0·05 M-potassium phosphate buffer (pH 6·8) at 66°C for 10 hr. All filters were washed, treated with RNase and rewashed as usual. The specific activity of the DNA varied from 411 cts/min/μg (5 μg DNA on the filter) to 354 cts/min/μg (234 μg DNA on the filter); that of the RNA was 280,433 cts/min/μg.

irreversible adsorption of DNA has not been exceeded. Further, as evidenced by the comparative constancy of the plateau values, there is no numerically significant decline in the availability of the DNA for hybridization as increasing amounts are fixed per filter.

(g) *A comparison of kinetics and plateau stability between liquid and immobilized DNA hybridization*

Figures 3 through 6 give some typical outcomes of hybridizations carried out at various input ratios and amounts of RNA and DNA. We may first focus attention on the results obtained with immobilized DNA. Comparing Figs 3 and 4 reveals, not

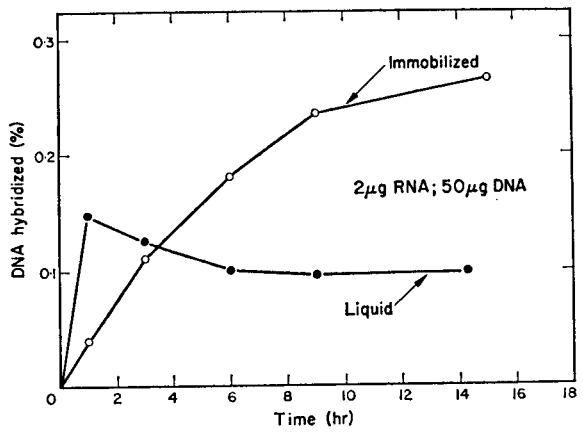

FIG. 3. Hybridizations with DNA in solution and immobilized.

Immobilized-DNA technique. DNA filters containing 50 μg DNA were prepared using 6×SSC and immersed in 5 ml. of a 6×SSC solution containing 2 μg RNA. Hybridization was carried out at 66°C without shaking. Purification of the hybrids is described in Materials and Methods.

Liquid technique. Reaction mixtures containing 50 μg DNA and 2 μg RNA in 1·5 ml. were made up in 6×SSC. The mixtures were held at 66°C, after which the hybrids were purified as described in Results section (c). DNA = 5000 cts/min/μg; RNA = 100,000 cts/min/μg.

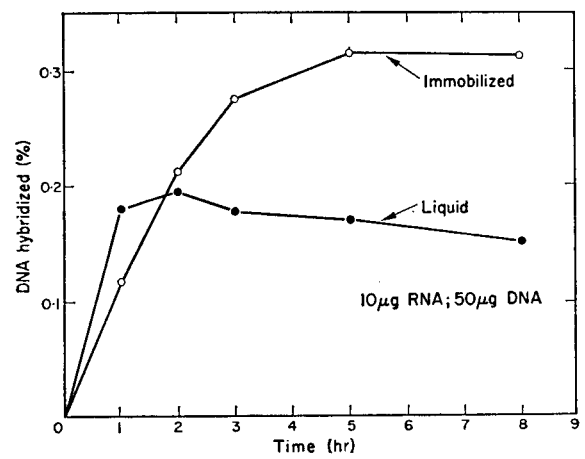

FIG. 4. Hybridizations with DNA in solution and immobilized. The procedures are the same as those described in the legend for Fig. 3, except that 50 μg DNA was hybridized with 10 μg RNA.

unexpectedly, that the rate of approach to the plateau is influenced by the concentration of RNA. At 10 μg of RNA, the hybridization reaches the expected plateau in five hours, a value not attained in 15 hours at 2 μg. On the other hand (Figs 5 and 6), the kinetics of complex formation is very slightly influenced by increasing the amount of DNA fixed to the membrane.

Comparison of these findings with the results obtained with DNA in solution dramatically illustrates the advantages of using immobilized DNA. We see (Fig. 3) that at the lower input of RNA the plateau is never reached in the liquid hybridizations. At a level of 50 μg RNA (Fig. 6), the saturation plateau is almost attained within a few hours; but this is then followed by a decomposition of formed hybrid, a

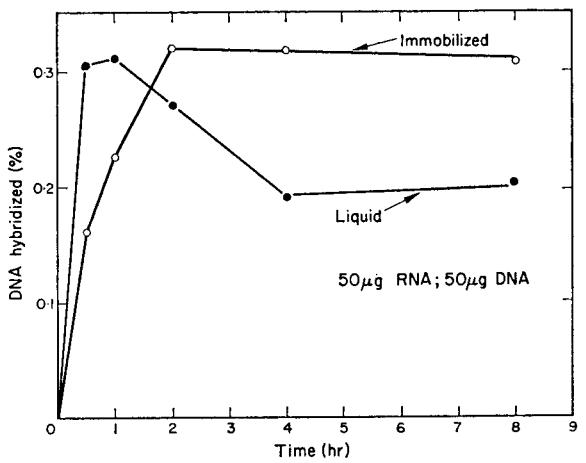

FIG. 5. Hybridizations with DNA in solution and immobilized. The procedures are the same a those described in the legend for Fig. 3, except that 50 μg DNA was hybridized with 50 μg RNA

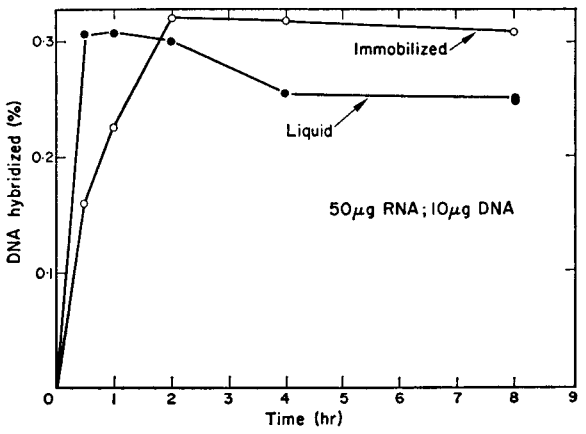

FIG. 6. Hybridizations with DNA in solution and immobilized. The procedures are the same as those described in the legend for Fig. 3, except that 10 μg DNA was hybridized with 50 μg RNA.

phenomenon reported by Nygaard & Hall (1964). In general, the loss of hybrids formed in liquid increases in severity with the concentration of DNA present. This phenomenon is effectively suppressed by immobilization of the DNA. At high levels of DNA per filter (50 μg and greater) and low ionic strength (2×SSC) some loss of hybrid is detected in the immobilized hybridizations. However, it is much less than that observed in annealing to DNA in solution and is negligible unless the hybridization is prolonged excessively.

4. Discussion

(a) *Some general precautions in hybridization experiments*

Unless care is exercised, basic proteins can occur as contaminants of DNA and RNA preparations. We have already pointed out that their presence can generate considerable difficulty by virtue of their ability to adsorb RNA, and the efficiency with which the resulting complex is retained by the filter. Nucleic acid preparations purified on

MAK columns can contain enough methylated albumin to cause this sort of trouble. It can be avoided by exhaustive washing of the columns before use.

It may be useful to others if we explicitly record here some of the other complications which can be encountered in carrying out hybridizations and the precautions we have adopted as a consequence. In most instances they center on the components of the system, and the details are best discussed in terms of the individual reactants and the steps involved.

DNA preparations

The DNA used must be completely available for hybridization if saturation plateaux are to be interpreted with confidence. It follows that the denaturation of the DNA employed must be shown to be complete.

A difficulty commonly encountered with DNA preparations is contamination with ribonuclease, a protein well known for its ability to bind to DNA and for its resistance to inactivation. The usual preparative procedures rarely provide DNA completely free of ribonuclease. Passage through an MAK column is extremely helpful since RNase remains behind. A useful alternative or supplement is to incubate the DNA with pronase to destroy residual RNase activity. The pronase can then be removed by deproteinization with phenol. It is important here first to subject the pronase to "self-digestion" to remove any nucleases it might contain. In any event, no DNA preparations should be employed which have not been assayed for ribonuclease activity for the time periods and under the conditions employed for hybridization.

RNA preparations

Assay for and removal of nuclease activity is, of course, equally important in the case of RNA. A frequently encountered difficulty is contamination with DNA fragments resulting from incomplete removal or digestion. This is true for RNA labeled with either ^{32}P or uridine, since many cells can convert the latter to thymine. It is a particularly serious complication with material containing glucosylated DNA (e.g., cells infected with T2, T4, or T6 bacteriophages) because these types of DNA are much more resistant to enzymic digestion. The DNase treatment must be continued until the residue of radioactive material stable in alkali is at an acceptable level. Even small amounts of contaminating labeled DNA can make the interpretation of many kinds of hybridization experiments impossible.

Enzymes

The RNase and DNase used must be assayed to be certain that each is free of significant contamination by the other. DNase can be readily freed of ribonuclease activity by column chromatography on DEAE (Polatnick & Bachrach, 1961). Deoxyribonuclease activity in RNase is readily destroyed by heating a solution adjusted to pH 5·0 to 90°C for 10 minutes. Assays for contaminating enzymes must be adequately sensitive and of a duration comparable to that employed in the relevant hybridization step. The use of radioactive polynucleotide as substrate makes it possible to achieve any desired level of sensitivity in the assay for contaminating activity.

Enzymic elimination of unpaired RNA

One of the most common pitfalls stems from the use of commercial RNase without checking for DNase contamination. No resistant plateaux of hybrid are observed if

DNase activity is detectable. The other is the failure to treat the hybrid under conditions specified for stability. Although stability may vary, in our experience temperatures above 37°C and salt concentrations below 0·25 M should be avoided. We obtain reproducibly an absolutely resistant hybrid fraction when enzyme treatments are carried out with 10 μg of RNase per ml. at 30°C in 0·3 M-NaCl. Wherever possible, internal controls of free RNA appropriately labeled with a radioactive isotope should be included, in order to be certain that the ribonuclease is functioning properly.

(b) *Methods of hybrid detection and assay*

Of the available methods of hybrid detection, two permit the actual isolation of the complex and allow its further characterizations. These involve the use of equilibrium density-gradients of CsCl or Cs_2SO_4, and the more recently developed chromatographic separations on MAK columns (Hayashi, Hayashi & Spiegelman, 1965). The latter method has the advantage in both capacity and convenience. Further, it has been shown in the same study that the column fractionates hybrids on the basis of the ratio of RNA to DNA in the complex.

The agar columns (Bolton & McCarthy, 1962) and nitrocellulose columns (Bautz & Hall, 1962) are particularly useful for preparative fractionation of particular classes of complementary RNA. The membrane filter is clearly the method of choice when large numbers of assays for complementarity are required.

(c) *Annealing with dissolved* versus *immobilized DNA*

The experiments reported here clearly establish that hybridizations with immobilized DNA are superior, conferring certainty and accuracy by avoiding DNA–DNA interactions. Hybridizations at elevated temperatures ($>$ 50°C) in liquid which are not monitored by a kinetic analysis are likely to be in error. If they are extended, they are almost certain to be low estimates.

The introduction by Nygaard & Hall (1963) of membrane filters for hybrid detection greatly expanded the number of samples it was feasible to analyze. The use of membrane-fixed DNA both for annealing and detecting the hybrid product generates still other advantages of convenience.

The technique is inherently flexible and can be adapted to almost any volume. For example, one need not use the entire filter, since after fixing a specified amount of DNA, circular subsections of known area can be punched out and employed. Further, more than one filter can be placed in the same hybridization mixture, and if desired, individual ones removed at suitable intervals and analyzed. Alternatively, a loaded filter can be left in for a fixed time, removed and replaced by a new one to allow further hybridization, permitting a simple search for heterogeneity in hybridizability. In addition, with the DNA immobilized, a wide temperature range is available uncomplicated by DNA renaturation. One can thus study the hybridization of molecules possessing high degrees of secondary structure and which require higher temperatures for annealing analysis.

The RNA hybridized to the fixed DNA can be recovered for further analysis by elution at elevated temperatures and low ionic strength. Finally, the combined use of RNase and the membrane-immobilized DNA makes it possible to push the "noise" level down easily to almost any desired level and it does not require extraordinary effort to achieve levels corresponding to 0·003% of the input RNA.

It may be noted in conclusion that this method has been successfully employed (Ritossa & Spiegelman, 1965) to establish that the "nucleolar organizer" segment of the X-chromosome of *Drosophila melanogaster* contains the DNA complements of ribosomal RNA. The experiments required that 25% differences in saturation plateaux be reliably detected at levels of 0·3% of the DNA input. The method was found adequate.

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